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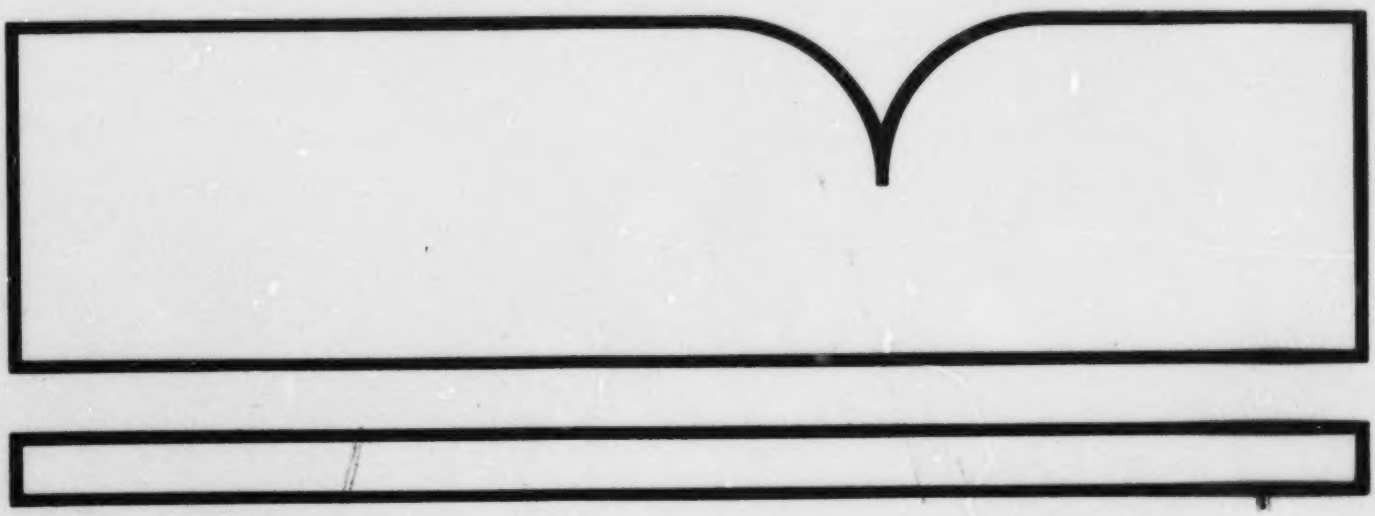

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16. ABSTRACT

Rat-embryo explants removed on day 10.5 of gestation were cultured for 48 hr in various concentrations of $HgCl_2$ CH_3HgCl or glutathione. Dose-related dysmorhogenesis and growth retardation occurred with increasing concentrations of $HgCl_2$ (1-10 uM). Increasing concentrations of CH_3HgCl (3-100 um) produced a similar pattern of embryonic effects. The rat-serum incubation medium had no detectable level of reduced glutathione (0.02 mM) and only 0.12 mM total sulphydryl groups. Reduced glutathione (60-300 uM) added to the incubation medium was relatively non-toxic. The addition of exogenous glutathione to culture medium containing $HgCl_2$ partially antagonized the embryonic growth retardation and prevented most of the embryolethality observed in cultures to which only $HgCl_2$ had been added.

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IN VITRO STUDY OF EMBRYOTOXIC AND DYSMORPHOGENIC EFFECTS OF MERCURIC CHLORIDE AND METHYLMERCURY CHLORIDE IN THE RAT*

K. T. KITCHIN, M. T. EBRON and D. SVENDSGAARD
Experimental Biology Division, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA

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Abstract—Rat-embryo explants removed on day 10.5 of gestation were cultured for 48 hr in various concentrations of $HgCl_2$, CH_3HgCl or glutathione. Dose-related dysmorphogenesis and growth retardation occurred with increasing concentrations of $HgCl_2$ (1–10 μM). Increasing concentrations of CH_3HgCl (3–100 μM) produced a similar pattern of embryonic effects. The rat-serum incubation medium had no detectable level of reduced glutathione (< 0.02 mM) and only 0.12 mM total sulphydryl groups. Reduced glutathione (60–300 μM) added to the incubation medium was relatively non-toxic. The addition of exogenous glutathione to culture medium containing $HgCl_2$ partially antagonized the embryonic growth retardation and prevented most of the embryolethality observed in cultures to which only $HgCl_2$ had been added.

INTRODUCTION

Human prenatal exposure to methylmercury can cause lesions of the central nervous system and abortions, as was seen in outbreaks of mercury poisoning in Japan and Iraq. These two outbreaks have been attributed to contaminated fish and to grain coated with a methylmercury pesticide, respectively (Matsumoto, Koya & Takeuchi, 1965; Snyder, 1971). Extensive studies with mice (Fuyuta, Fujimoto & Hirata, 1978; Su & Okita, 1976), rats (Fuyuta *et al.* 1978) and hamsters (Gale & Ferm, 1971) showed an increased incidence of cleft palate, exencephaly, oedema, retarded growth, hydrocephaly and hydronephrosis in term foetuses treated with various doses of methylmercury during organogenesis.

In contrast, data on the embryotoxic and dysmorphogenic effects of the inorganic forms of mercury are relatively scarce. Skerfving & Vostal (1972) and Lomholt (1928) reported cases of spontaneous abortion in women being treated with mercuric chloride for syphilis. The ingestion of 40 mg mercuric chloride/kg brought about the abortion of a normal human foetus 5 cm long (Afonso & deAlvarez, 1960). Murakami (1972) cited a study in which ocular and skeletal defects and reduced body weights were observed in rats treated with inorganic mercury.

Gale & Ferm (1971) and Gale & Hanlon (1976) found that both organic mercury and mercuric chloride were embryotoxic when administered at dose levels that caused substantial teratogenicity. Other animal studies comparing the effects of organic and inorganic mercury have shown that organic mercury crosses the rat placenta twelve times faster and accumulates in the foetuses five to seventeen times faster than inorganic mercury (Garrett, Garrett & Archdeacon, 1972; Kelman, 1977; Mansour, Dyer, Hoffman *et al.* 1973). The different gestational times of administration of the mercury compounds complicate interpretation of the experimental evidence. When administered early in gestation, inorganic mercury reaches the embryo, whereas later the developing chorioallantoic placenta becomes a more efficient barrier to the transmission of this xenobiotic compound (Schulert, Glasser, Stant *et al.* 1969).

Heavy metals can form reversible complexes with organic ligands, particularly sulphydryl groups. These complexes play an important role in the distribution and excretion kinetics of the metals. Glutathione amd metallothionein are examples of important maternal sulphydryl-containing molecules that function as endogenous chelators.

The study described here compared the concentration-response relationship for the embryotoxic and dysmorphogenic effects of methylmercury chloride and mercuric chloride using *in vitro* whole-embryo cultures. Thus maternal–placental transport and the metal-binding properties of maternal glutathione or metallothionein were eliminated. For these studies, it was established that the incubation medium (whole rat serum) contained no detectable levels of glutathione and only 0.12 mM total sulphydryl. As mercuric chloride has a high affinity for sulphydryl groups, glutathione was added to the culture system to determine whether this compound could counteract the embryotoxicity and dysmorphogenicity caused by mercuric chloride. An attempt was also made to determine the most sensitive embryonic parameter for these metal toxicants.

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EXPERIMENTAL

Whole-embryo culture

Male and female Sprague–Dawley rats of the CD strain (Charles River Breeding Laboratories, Kingston, NY) were housed together overnight, and the following morning vaginal smears were made. Females with sperm-positive smears were considered to be at day 0.5 of gestation. On day 10.5 *post coitum*, pregnant rats were killed by guillotine and a laparotomy was performed to expose the gravid uterus. Under aseptic conditions, conceptuses were removed from the uterus and collected in Petri dishes containing Trowell's T8 medium. The decidual tissue and Reichert's membrane were removed by the technique described by Sanyal & Wiebke (1979). Whenever possible, embryos harvested from the same dam were put in different treatment groups for culturing.

The culture medium was prepared from blood which was drawn from the dorsal aorta of 90-day-old male CD rats, immediately centrifuged, allowed to stand at room temperature for 1 hr and then recentrifuged for 30 min. The serum was stored at −20°C until needed (usually within a 2-wk period). Prior to its use, the serum was thawed, filtered and heat-inactivated at 56°C for 30 min (New, 1978; Steele & New, 1974). The serum was allowed to cool and 50 U penicillin/ml and 50 μg streptomycin/ml were added.

Each explant was cultured for 48 hr in a 30-ml serum bottle containing 5.0 ml culture medium and a mixture of gases (5.0% CO_2: 95% air on day 10.5, followed by 5.0% CO_2: 40.0% O_2: 55.0% N_2 on day 11.5). Each culture bottle was rotated at 60 rpm on a roller culture assembly in a 37°C incubator. At the end of the culture period, explants were removed from the culture vessel and observed microscopically. The explants were evaluated for growth and differentiation using criteria that included increased yolk-sac diameter, crown–rump length, head length and number of somites. Embryos without a beating heart were classified as dead. Dead embryos were excluded from morphological scoring. Morphogenesis was scored as normal or abnormal in comparison with untreated controls. After homogenization with ultrasound, embryos and yolk sacs were stored at −20°C for later quantification of DNA (Boer, 1975) and total protein (Lowry, Rosebrough, Farr & Randall, 1951). Levels of glutathione and total sulphydryl groups in the serum from male rats (incubation medium for the embryo explants) or in the liver homogenates from 14-day-pregnant rats were determined by the method of Sedlak & Lindsay (1968).

All test compounds were added to the serum at the beginning of the incubation period. A mercuric chloride atomic absorbance standard solution (1 mg/ml) was obtained from Fisher Scientific Company (Fairlawn, NJ) and diluted to prepare a 0.5 mM stock solution. Stock solutions of methylmercury (ICN Pharmaceuticals, Cleveland, OH) were also prepared in sterile distilled water in concentrations of 0.5 and 5.0 mM. Reduced glutathione was purchased from Calbiochem (La Jolla, CA) and fresh 3.0 mM stock solutions were prepared on the day of use. Embryos grown in undiluted serum or in serum containing up to 10% distilled sterile water grew and differentiated similarly.

Quantitative data are presented as means ± SEM for five or more independent observations. Statistical significance was determined by analysis of variance procedures comparing treatment groups with controls run on the same day (Snedecor & Cochran, 1967). Enumerative data were evaluated statistically using a chi-square exact test (Goldstein, 1964) and are reported as a percentage occurrence.

RESULTS

Rat embryos exposed *in vitro* to 1–3 μM-$HgCl_2$ grew at a rate similar to the controls. At a concentration of 4 μM-$HgCl_2$, embryonic protein and DNA content were significantly reduced (by 23 and 36%, respectively; Fig. 1). At a concentration of 6 μM-$HgCl_2$, explanted rat embryos grew to approximately the same extent as at 4 μM. At 6 μM-$HgCl_2$, significant reductions were observed in the somite number and crown–rump length (Fig. 2) and in head length. At

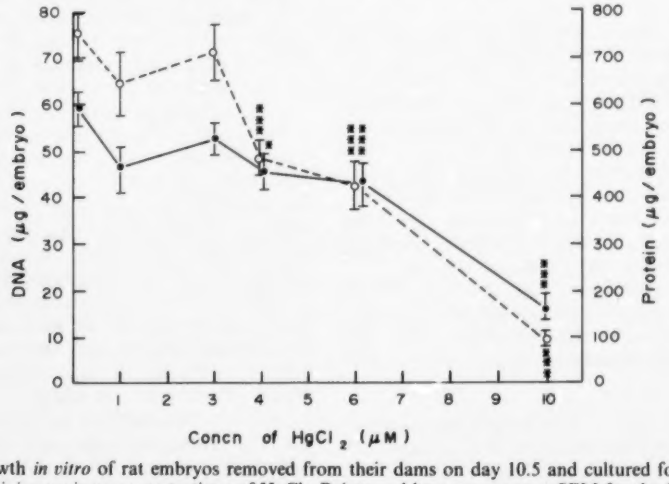

Fig. 1. Growth *in vitro* of rat embryos removed from their dams on day 10.5 and cultured for 48 hr in media containing various concentrations of $HgCl_2$. Points and bars are means ± SEM for the final DNA (○) and protein (●) content of 10–24 embryos (*$P < 0.05$; ***$P < 0.001$).

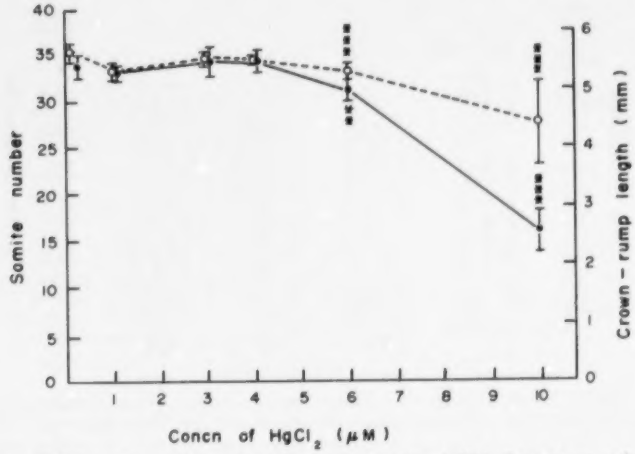

Fig. 2. Mean (±SEM) somite number (○) and crown–rump length (●) of 10–24 rat embryos explanted on day 10.5 and cultured for 48 hr in media containing various concentrations of $HgCl_2$ (**$P < 0.01$; ***$P < 0.001$).

this concentration, 80% of the embryos possessed a functioning circulatory system, while 10 μM-$HgCl_2$ killed ten out of 11 explants. At 10 μM-$HgCl_2$, the highest concentration used, extreme growth retardation was evident, particularly in respect of embryonic DNA and protein content and crown–rump length.

With CH_3HgCl, significant growth effects did not occur at concentrations of 3 or 10 μM. Thus, *in vitro*, CH_3HgCl is several times less toxic than the inorganic form of mercury, $HgCl_2$. Rat embryos exposed to 30 μM-CH_3HgCl grew and differentiated significantly less than did controls exposed to an equal volume of distilled water (Fig. 3). Severe growth inhibition occurred at a concentration of 60 μM and the embryos could not be scored for crown–rump length or somite number. At this concentration, all the embryos were dead. The inhibitory effect of 100 μM-CH_3HgCl on *in vitro* embryonic protein and DNA synthesis was more pronounced than the effects at 60 μM.

When experimental animals are exposed to metal ions *in vivo*, endogenous chelators, such as glutathione and metallothioneins, can alter the pharmacokinetics and toxicity of the metal. As most of the glutathione in blood is located inside the red blood cells (Altman & Dittmer, 1971), heat-inactivated rat serum should contain little or no glutathione. Thus, the concentrations of glutathione and total sulphydryl groups in the *in vitro* embryo incubation medium were compared with those found in maternal liver on gestation day 14. No detectable glutathione was found in the culture serum, while in maternal liver a concentration of 5.71 ± 0.12 μmol glutathione/g liver (mean ± SEM for 12 independent observations) was observed. The assay used could detect

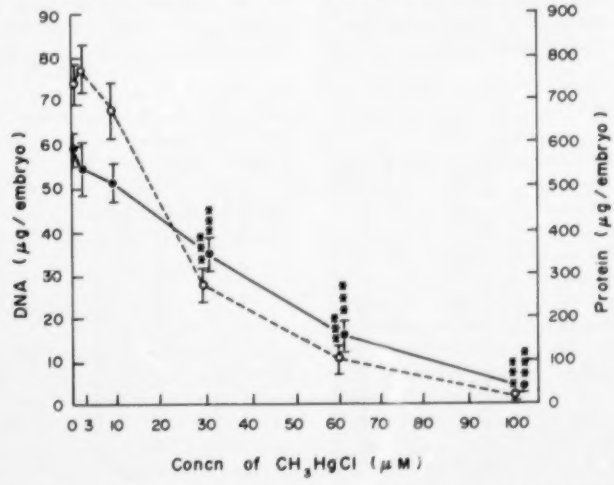

Fig. 3. Means (±SEM) for 5–21 determinations of DNA (○) and protein (●) content of rat embryos exposed *in vitro* to various concentrations of CH_3HgCl or (for controls) to equal volumes of distilled water (***$P < 0.001$).

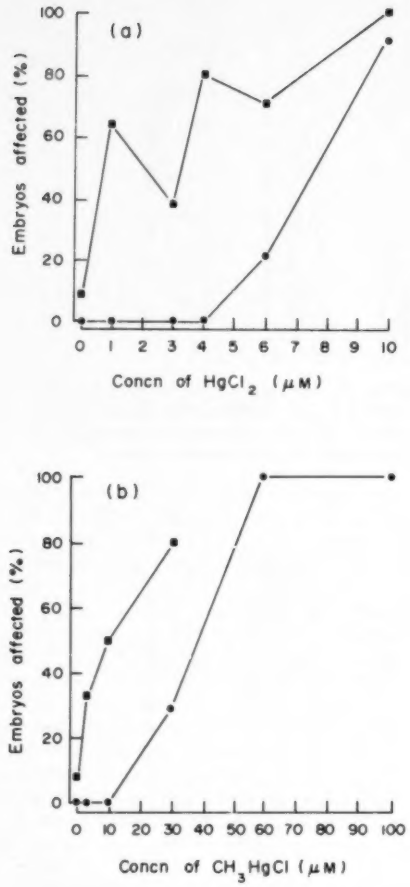

Fig. 8. Percentages of abnormal (■) and dead (●) embryos in cultures of explanted rat embryos exposed for 48 hr to different concentrations of (a) $HgCl_2$ (more than ten explants/treatment) or (b) CH_3HgCl (five explants exposed to 100 μM; nine or more/group for other exposure levels). Embryos with no heartbeat were classified as dead and excluded from morphological assessment.

(1974) found that ip administration of 2–3 mg mercuric acetate/kg increased the rate of resorptions and terata whereas, in previous studies, 2 mg mercuric acetate/kg had caused only a limited number of adverse effects (Gale & Ferm, 1971). Again in hamsters, 4 mg mercuric acetate/kg was embryotoxic when given iv or ip but not when administered sc (Gale, 1974; Gale & Ferm, 1971). Doses of mercuric acetate between 8 and 20 mg/kg were clearly teratogenic and embryolethal (Gale, 1974 & 1981). From these hamster studies, it seems that approximately 3 mg mercuric acetate/kg is the *in vivo* dose at which embryonic resorptions and terata occur. This dose corresponds to 9 μM, or approximately twice the concentration (4 μM) at which $HgCl_2$ exhibited adverse growth-retardation effects in rat whole-embryo culture. This dose and concentration comparison assumes that the *in vivo* volume of distribution and density of the intact animal are similar to those of the culture medium. A smaller volume of distribution will raise the serum level of a compound while excretion processes will lower the serum levels. However, if morphology alone is used as the criterion, adverse effects occurred at 1 μM-$HgCl_2$, the lowest concentration tested *in vitro*.

In our studies, it was found that heat-inactivated rat serum contained no detectable level of reduced glutathione. This small sulphydryl-containing tripeptide influences the *in vivo* pharmacokinetics and toxicity of exogenous metals. Richardson & Murphy (1975) reported that *in vivo* glutathione depletion reduced $HgCl_2$ accumulation in the rat kidney, brain, red blood cells and liver. Other investigators (Magos, Clarkson & Allen, 1978) administered glutathione and CH_3HgCl to rats and observed that the levels of CH_3HgCl were decreased in whole blood and increased in the liver compared with those following saline and CH_3HgCl administration. Glutathione is an example of an *in vivo* chelator that is not present in *in vitro* embryo culture. Without such chelators, there is the possibility that *in vitro* embryo culture will show excessive metal toxicity and will thus not predict accurately the *in vivo* response.

The data in this report as well as those from two other studies (Mirkes, Fantel, Greenaway & Shepard,

Table 1. Concentration-related order in which conceptus parameters were adversely affected by toxicants

Relative degree of response to toxicant	Order of degree of response of parameters to:				
	$HgCl_2$*	CH_3HgCl*	Glutathione*	Acrolein†	Phosphoramide mustard‡
		Yolk sac			
More responsive	Protein, DNA	Protein, DNA	Diameter	Diameter	ND
Less responsive	Diameter	Diameter	DNA, protein	DNA, protein	ND
		Embryo			
Most responsive (1)	% abnormal	% abnormal	% abnormal	DNA, somites	% abnormal
Intermediate (2)	Protein, DNA	§	Crown–rump length, head length, lethality, somites	Crown–rump length	Protein
Intermediate (3)	Crown–rump length, head length, somites	§		Head length, protein	Crown–rump length
Least responsive (4)	Lethality	Lethality		Lethality	Somites

ND = No data given

*Data from this study.
†From Schmid *et al.* 1981.
‡From Mirkes *et al.* 1981.
§All variables apart from those identified responded similarly.

1981; Schmid, Goulding, Kitchin & Sanyal, 1981) may be used in an attempt to pinpoint the most sensitive yolk-sac or embryo parameter for detecting toxicity. Table 1 summarizes the order in which conceptus parameters were significantly ($P < 0.05$) affected by increasing doses of toxicants. With both $HgCl_2$ and CH_3HgCl, yolk-sac protein and DNA contents were decreased at the lower doses while at higher concentrations decreases in yolk-sac diameters were noted. However, acrolein (Schmid *et al.* 1981) and glutathione showed the opposite pattern.

The most sensitive embryonic parameter appeared to be the percentage of abnormal embryos (where abnormalities occurred). Apart from this, the five chemicals that were compared produced a different order of sensitivity in embryonic toxicity parameters. We therefore conclude that all embryonic effects should be measured, since no single parameter appears to respond reliably and selectively to low doses of different chemical toxicants.

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